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We have studied the effect of vitamin D analogs on growth of various breast cancer cell lines in vitro as well as in vivo and have identified the 19-nor-hexafluoride analogs as being particularly potent in their activity. Furthermore, we have found that vitamin D_3 analogs can synergize with Taxol in inhibiting breast cancer. We have recently found that troglitazone, a synthetic ligand of the peroxisome proliferator/activated receptor γ (PPARγ) can inhibit the growth of breast cancer cells and this PPARγ ligand with All-trans-retinoic acid can induce apoptosis of breast cancer cells. Furthermore, we have looked for genetic alterations of breast cancer including identification of alterations of the cyclin-dependent-kinase inhibitor genes including $p27^{KIP1}$ and the $p16^{INK4A}$ genes.

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INTRODUCTION

The following provides descriptions of our vitamin D, retinoid, trogliterazone studies as well as our molecular biology studies in breast cancer.

I. Vitamin D_3 and Breast Cancer

I.1) 19-nor-Hexafluoride Analogue of Vitamin D3: A novel class of potent inhibitors of proliferation of human breast cell lines

Authors: Koike M, Elstner E, Campbell M, Asou H, Uskokovic M, Tsuruoka N, and Koeffler HP

Breast cancer cells express vitamin D3 receptors and 1,25-dihydroxyvitamin D_3 suppressed growth of these cells. We have synthesized six novel vitamin D_3 analogues to identify those with expanded capacity to inhibit the proliferative ability of breast cancer cells. These analogues incorporated many of the structural motifs shown previously to have antiproliferative activities, and LH [1,25-$(OH)_2$-16-ene-23-yne-26,27-F_6-19-nor D_3] was the most potent analogue, suppressing at 10^{-11}M greater than 50% clonal proliferation (ED_{50}) of the MCF-7 and SK-BR-3 breast cancer cells, increasing the proportion of MCF-7 cells in the G_0-G_1 phase, and decreasing those in the S phase of the cell cycle. Pulse-exposure studies showed that a 3-day-exposure to LH (10^{-7}M) in liquid culture was adequate to achieve a 50% inhibition of MCF-7 clonal growth in soft agar in the absence of the analogue, suggesting that the growth inhibition mediated by LH is irreversible. The cyclin-dependent kinase inhibitor known as $p27^{KIP1}$ helps regulate the cell cycle and can mediate growth arrest in response to extracellular growth inhibitors. The analogue LH (10^{-7}M) induced elevated expression of $p27^{KIP1}$ in MCF-7 and SK-BR-3 cells. Taken together, these results indicate that LH is an extremely potent vitamin D3 analogue markedly inhibiting clonal growth of MCF-7 and SK-BR-3 cells. Taken together, these results indicate that LH is an extremely potential vitamin D3 analogue markedly inhibiting clonal growth of MCF-7 and SK-BR-3 cells with concomitant cell cycle arrest at G_0-G_1 and increased expression of $p27^{KIP1}$. Compound LH is worthy of in vivo analysis for possible future clinical trials.

Submitted for publication, 1997.

I.2) Combined effect of vitamin D3 analogs and Paclitaxel on the growth of the MCF-7 breast cancer cells in vivo

Authors: Koshizuka K, Koike M, Asou H, Cho S, Stephen T, Rude R, Binderup L, Uskokovic M, and Koeffler HP

Vitamin D_3 analogs and paclitaxel (Taxol) are able to inhibit growth of a variety of malignant cells. We examined the ability of three vitamin D_3 compounds to inhibit growth of a human mammary cancer (MCF-7) in BNX triple immunodeficient mice either alone or with Taxol. Vitamin D_3 analogs were 1,25$(OH)_2D_3$ (code name, Compound C), 1,25(OH)2-16-ene-23-yne-nor-26,27-F_6-D_3 (Compound LH) and 24a,

26a, 27a,-trihomo-22,24-diene-1,25$(OH)_2D_3$ (EB1089). At the doses chosen, the antitumor effect of vitamin D3 analogs alone was greater than that of Taxol alone, and an additive effect was observed when vitamin D3 analog and Taxol were administered together. EB1089 was the most potent compound; and the EB1089 plus Taxol was the most potent combination decreasing the tumor mass nearly 5-fold compared to control. Except for the Taxol group, each experimental cohort weighed $\leq$ 20% of the control group. None of the animals became hypercalcemic. Their complete blood counts, serum electrolyte analysis as well as their liver and renal functions were all fairly similar and within the normal range. In summary, this combination of a vitamin D_3 analog and Taxol has the potential to be a therapy for breast cancer.

I.3) **Effect of vitamin D3 analog, paclitaxel, and cisplantin on the growth of MCF-7 human breast cancer cells in vivo**

Authors: Koshizuka K, Koike M, Kubota T, Said J, Binderup L, and Koeffler HP

Vitamin D_3 analogs, paclitaxel (Taxol) and cisplantin (CDDP) inhibit growth of a variety of malignant cells. We examined the ability of a novel 20-epi-vitamin D_3 analog (code name, CB1093), Taxol and CDDP either alone or in combination to inhibit growth of a human mammary cancer (MCF-7) growing in BNX triple immunodeficient mice. Control animals demonstrated infiltrating poorly differentiated adenocarcinomas. At the does chosen, the antitumor effect of Taxol alone was greater than that of either CB1093 + Txol or CB1093 + CDDP + Taxol were administered together. The combination of CB1093 + Taxol + CDDP was most potent, decreasing tumor weight by nearly 83% compared to control tumors and producing extensive necrosis of the remaining tumor mass. No additive effect on reduction of tumor weights occurred by combing either CB1093 + CDDP or Taxol + CDDP compared to Taxol alone. For all cohorts, their complete hematopoietic blood counts, serum electrolyte analyses including serum calciums as well as their liver and renal functions were within the normal range. Extensive histological analyses of the liver, spleen, kidney, bone marrow, skin and subcutaneous fat pad from these mice showed no changes from the control mice. In summary, combined therapy with a potent vitamin D_3 analog, Taxol, and CDDP may hold promise in the treatment of patients with breast cancer.

Submitted for publication, 1997.

I.4) **Potent Vitamin D_3 analogs and their 24-oxo metabolites equally inhibit clonal proliferation of a variety of cancer cells, but have differing molecular effects**

Authors: Campbell MJ, Reddy G, Koeffler HP.

The seco-steroid hormone, 1α, 25 dihydroxyvitamin D3 (1α,25$(OH)_2D_3$) binds to a specific nuclear receptor that acts as a ligand inducible transcription factor. The resulting genomic effects include partial arrest in G_0/G_1 of the cell cycle and induction of differentiation; these effects have been observed in various types of cancer. Recently, we produced enzymatically the natural 24-oxo metabolites of

$1\alpha,25(OH)_2D_3$ and two of its potent synthetic analogs ($1\alpha,25\text{-}(OH)_2\text{-}16\text{-ene-}D_3$ and 1α, $25\text{-}(OH)_2\text{-}20\text{-epi-}D_3$) using a rat kidney perfusion system. We have found that 24-oxo metabolites of both $1\alpha,25(OH)_2D_3$ and its analogs have either the same or greater antiproliferative activity against various cancer cells as their parental compounds. Notably, two cell lines (DU-145 (prostate cancer) and MDA-MB-436 [breast cancer]) that were extremely resistant to the antiproliferative effects of vitamin D_3 analogs displayed greater sensitivity towards the 24-oxo metabolite of the vitamin D_3 analog. Similarly, the 24-oxo metabolites had the capacity to induce differentiation and apoptosis and to diminish the proportion of cells in S phase. Most interestingly, while the analog 1α, $25\text{-}(OH)_2\text{-}20\text{-epi-}D_3$ induced expression of BRCA1 in MCF-7 breast cancer cells; its 24-oxo metabolite dramatically suppressed BRAC1 expression. Thus, we have shown for the first time that the various biological activities produced by the hormone $1\alpha,25(OH)_2D_3$ and some of its analogs may represent a combination of actions by hormone $1\alpha,25(OH)_2D_3$ and its natural 24-oxo metabolites.

J of Cell Biochem, 66(3):413-425, 1997.

I.5) **Preadipocytes stimulate breast cancer cell growth**

Authors: Chamras H, Baggs D, Elstner E, Koeffler HP, and Heber D.

Local growth-regulatory factors elaborated by mammary stromal cells including fibroblasts, preadipocytes and adipocytes may influence breast cancer cell growth. We studied the effect of the parent Swiss 3T3 mouse fibroblast cell line and its subline 3T3-L1 predadipocytes cell line, which both were used as feeder layer, on clonal growth of human breast cancer estrogen-(ER)positive MCF-7 and ER-negative MDA-MB-231 and MDA-MB-436 cell lines. Our study showed that proliferating 3T3-L1 cells stimulated the clonal growth of MCF-7, MDA-MB-231 and MDA-MB-436 cells by two-fold, 43% and 60%, respectively. However, confluent 3T3-L1 cells lost this stimulatory ability. In contrast, the differentiated mature adipocytes significantly inhibited clonal growth of breast cancer cell lines. The identification of ligands and target receptors, involved in this process is under investigation.

Submitted for publication, 1997.

I.6) **The 16-end vitamin D_3 analogs**

Authors: Uskokovic M, Studzinski G, Gardner J, Reddy G, Campbell MJ, and Koeffler HP.

Numerous 16-ene vitamin D analogues were investigated as potential anticancer agents. Several structural modifications have been uncovered that contribute to the improvement in the stimulation of HL-60 cells differentiation, the inhibition of HL-60 cells proliferation and the reduction of calcemic properties *in vivo.* They include the introduction of 16-, 22E-, 23E, and 23Z-double bonds, 23-triple bond or 22R-allene, and substitution of C26 and C27-hydrogens with florine or methyl groups. The

biggest gains have been achieved by combination of the 16-double bond with 23-double or triple bond and 26-trifloro or 26,27-hexaflouro substitution patterns. Separately, the combination of the 16-double bond with 22R-allene has produced a highly active analog. In respect to modifications in the ring A, the high activities in cell differentiation and inhibition of cell proliferation with significant reduction of calcemic properties were observed in the 1α-flouro, 3-desoxy, and 19-nor series. It was also shown that the lack of the 1α-hydorxy group can overcome by an optimized modification in the ring D and the side chain; 25(OH)-16,23E-diene-26,27-F6D3 is fully active in HL-60 cell differentiation assay with only minimal effects on the cellular calcium homeostasis.

Cur Pharmaceut Des, 3:99-123, 1997.

I.7) **Towards therapeutic intervention of cancer by vitamin D compounds**

Authors: Campbell M, Koeffler HP.

We (Campbell M, Koeffler HP) reviewed the use and function of novel vitamin D analogs for treatment of human malignancy including breast cancer.

Natl Cancer Inst, 89(3)182-186, 1997.

I. 8) **Integrity of the 1,25-dihydroxyvitamin D3 receptor in bone, lung, and other cancers**

Authors: Miller CW, Morosetti R, Campbell M, Mendoza S, and Koeffler HP.

Differentiation and proliferation can be regulated in diverse cell types by 1,25-dihydroxyvitamin D_3. These effects derive from modulation of gene expression mediated by the interaction of 1,25-dihydroxyvitamin D_3 with the vitamin D receptor (VDR). The VCR is one of the nuclear hormone receptors. Because these transcription factors play a key role in growth control, some nuclear hormone receptors, such as retinoic acid receptor α, can be disrupted in cancer. With these alterations in mind, we looked for alterations of the VDR gene in a variety of cancers, including 68 osteosarcomas, 23 other sarcomas, 34 non-small cell lung cancers, and 44 cell lines representing many tumor types. Gross integrity of the VDR gene was examined on Southern blots probed with the coding region of the VDR cDNA. The presence of point mutations targeting VDR exons 2-7 was assessed by polymerase chain reaction-single=strand conformation polymorphism analysis and direct DNA sequencing. Two alterations were detected; direct DNA sequencing of these samples revealed one silent mutation in codon 9 and a base change in intron 3. These results suggest that mutations and rearrangement of the VDR do not play a role in the cancers studied.

Mol. Carcinog. 19:254-257, 1997.

II.) Retinoids, ligands for PPARγ and breast cancer

Authors: Elstner E, Koshizuka K, Williamson E, Hiroya A, Shintaku P, Said J, and Koeffler HP.

The peroxisome proliferator-activated receptor gamma (PPARγ), a member of the nuclear hormone receptor superfamily, can be activated through its synthetic ligand, the anti-diabetic drug trogliterazone (T). The retinoic acid receptor (RAR)-specific ligand, all-trans retinoic acid (ATRA) inhibits growth of breast cancer cells. Our aim was to investigate the effect of T and ATRA on proliferation, differentiation and apoptosis of breast cancer cells in vitro and in vivo. In contrast to PPARγ-negative human normal breast epithelial cells, fresh breast cancer and breast cancer cell lines prominently expressed PPARγ. In vitro, T inhibited the growth of PPARγ-positive MCF7 breast cancer cells and strongly upregulated lipid accumulation in these cells, but did not affect the normal breast epithelial cell line HB125. Furthermore, the combination of T with ATRA synergistically and irreversibly inhibited growth, as well as induced differentiation, apoptosis and down-regulated lipid accumulation in MCF7 cells. In vivo, T (100 mg/kg/day) significantly and non-toxically inhibited MCF7 tumor growth in triple immunodeficient mice during 9 weeks of treatment. Combined treatment with T and ATRA (7.5 mg/kg/day) caused massive apoptosis, necrosis, and fibrosis in MCF7 tumors. Taken together, the combination of ligands for PPARγ and RAR inhibited growth and induced differentiation and apoptosis of breast cancer cells in vitro and in vivo;and therefore, it may provide a novel, non-toxic and selective therapy for human breast cancers.

Submitted as an abstract to the Amer Assoc Cancer Res, 1997.

III. Molecular Biology of Breast Cancer

III.1) Molecular analysis of INK4 genes in breast carcinomas

Authors: Spirin K, Simpson J, Miller CW, and Koeffler HP

Cell cycle regulators have recently been implicated in oncogenic transformation of cells, including the cyclins active in the G1 phase of the cell cycle and their respective cyclin-dependent kinases (CDK) whose activities are regulated by a set of inhibitors of CDK (CDKI). Since CDKIs can inhibit cell proliferation, they many have a role as tumor suppressor genes. To determine if alterations of CDKI genes may be involved in tumorigenesis of breast cancer, we examined the mutational status of $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, $p19^{INK4D}$ CDKI genes in 36 primary breast carcinomas and 9 breast cancer cell lines using polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP), direct DNA sequencing, and Southern blot analysis. Furthermore, amplification of cyclin D1, D2, D3 genes were also examined in these samples. One mutation of $p15^{INK4B}$ gene occurred, resulting in change of aspartic acid to asparagine at codon 86. Since aspartic acid at this position is conserved between all four human and murine INK4 proteins, this missense mutation may have functional significance. The sample with a $p15^{INK4B}$ point mutation was accompanied by amplification of the cyclin D1 gene. A deletion

of the $p18^{INK4C}$ gene was found in a primary tumor. Three deletions of the $p16^{INK4A}$ gene and two deletions of the $p15^{INK4B}$ gene were found in the cell lines. Also, we found amplification of the $p16^{INK4B}$ and $p16^{INK4A}$ loci in a clinical sample as well as amplification of the $p19^{INK4D}$ in another sample, and amplification of the myeloperoxidase (MPO) gene in one cell line and two primary tumors. We suspect that a critical gene for breast cancer is amplified near the MPO gene. These data indicate that CDKI mutations are moderately rare in breast cancer and often associated with the simultaneous alteration of more than one cell-cycle regulatory gene.

Int J Oncol, 11:737-744, 1997.

III.2) **p 27KIP1 mutation found in breast cancer**

Authors: Spirin K, Simpson J, Takeuchi S, Kawamata N, Miller CW, and Koeffler HP.

The p27/Kip1 protein belongs to the recently identified family of proteins called cyclin-dependent kinase inhibitors. These proteins play an important role as negative regulators of cell cycle-dependent kinase activity during progression of the cell cycle. Since cyclin-dependent kinase inhibitors can inhibit cell proliferation, they may have a role as tumor suppressor genes. To determine whether p27 alterations may be involved in tumorigenesis, we examined its mutational status in 36 primary breast carcinomas and 9 breast cancer cell lines using PCR-single-strand conformational polymorphism, direct DNA sequencing, and Southern blot analysis. Southern blot analysis showed no homozygous deletions of the p27 gene in either the clinical samples or cell lines. Two point mutations were found in primary tumors. One represents a previously undescribed polymorphism at codon 142; another is a nonsense mutation at codon 104. The latter mutation was absent in the normal matched control sample, and, in addition, it was accompanied with the loss of heterozygosity (LOH) of a microsatellite marker in the vicinity of the p27 gene on chromosome 12p13. These data indicate that p27 mutations are a rare event in breast cancer, but may play an important tole in the development of a minority of these cancers. Furthermore, LOH analysis of the 12p13 locus revealed that an additional four of six matched DNA samples had LOH at 12p13 but did not have an alteration of the p27 gene, suggesting that another tumor suppressor gene is located on the short arm of human chromosome 12 which may be frequently involved in the pathogenesis of breast cancers.

Cancer Res 56:2400-2504, 1996

CONCLUSION

We have identified some particularly potent vitamin D analogs that inhibit the growth and induce differentiation of breast cancer cells. We have found that troglitерazone, a ligand for PPARγ synergizes with all-trans-retinoic acid in the inhibition of growth and induction of apoptosis of breast cancer cells. We have found that vitamin D analogs in combination with certain chemotherapeutic agents can enhance the effect of those chemotherapeutic agents, and finally; we have identified some of the molecular biology alterations of breast cancer.

REFERENCES

Please see body of grant; references cited after each section.